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Anxiolytic-Like Activity and Mode of Action of Lactomedin-2, an Oxytocin Receptor Agonist Peptide Derived from Human Lactoferrin

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Abstract

Lactomedin-2 (LM-2: CFQWQR), isolated from a trypsin digest of human lactoferrin as an ileum-contracting peptide, exhibited a weak affinity (Ki=62 μM) for the human oxytocin receptor (OT-R). LM-2 also exerted an anxiolytic-like activity in male mice at doses of 0.1 nmol/mouse (*icv*), 0.3 to 1 mg/kg (*ip*) and 30 mg/kg (*po*), as evaluated by the elevated plus-maze test. Furthermore, LM-2 exerted anxiolytic-like activity at a dose of 1.0 mg/kg when orally administered as an emulsion in 30% egg yolk. The anxiolytic-like activity of LM-2 after *icv* administration was blocked by L-371257, an antagonist of the OT-R. LM-2 was thus demonstrated to be an agonist peptide of the OT-R, the first to be derived from a natural protein other than preproxytocin. It is also the first example of an orally effective OT-R agonist peptide.

The anxiolytic-like activities of LM-2 were blocked by SCH58267, an antagonist of the adenosine A_{2A} receptor. It was also shown the first time that the anxiolytic-like activity of oxytocin was blocked by SCH58267. Furthermore, the anxiolytic activity of LM-2, as well as that of oxytocin, was blocked by bicuculine, an antagonist of the $GABA_A$ receptor. From these results it is concluded that the anxiolytic-like activities of LM-2 and oxytocin are mediated successively by the adenosine-A_{2A} receptor and GABA-$GABA_A$ receptor systems downstream of the OT-R.

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Introduction

The exogenous peptides that interact with the receptors for endogenous bioactive peptides have been isolated from the enzymatic digests of proteins of various origin such as blood, milk, eggs and plants which have not been regarded as precursors of bioactive peptides [1, 2].

The contractile responses of an isolated ileum preparation provide a convenient method for screening bioactive substances, since the receptors for various endogenous substances coupled to contraction are expressed in this system. Many kinds of endogenous bioactive peptides have been isolated in this way [3]. We have isolated bioactive peptides from the enzymatic digests of natural proteins, including bradykinin, neurotensin and complements C3a and C5a [4-8].

Lacroferrin is present not only in milk, but also in body fluids such as saliva, tears and pancreatic juice. It also is found in various tissues, including the brain [9]. The lactoferrin obtained from various animals has been shown to have certain physiological effects, such as antimicrobial and anti-inflammatory activities [10, 11]. Some of the physiological effects of lactoferrin have been ascribed to peptides released by proteases [2, 11].

From a trypsin digest of human lactoferrin, we obtained two ileum-contracting peptides, FKDCHLAR and CFQWQR, and named them lactomedins -1 and -2 respectively. Lactomedin-1 (LM-1) proved to be an agonist of the receptor of complement C5a (C5a-R) [12]. In this investigation, we found that lactomedin 2 has a weak affinity for the human oxytocin receptor.

Besides certain effects in females such as uterine contractions and lactation, oxytocin exerts central nervous system effects, such as anxiolytic-like and prosocial activities, irrespective of gender [13]. We therefore tested whether LM-2 exerted anxiolytic-like activity in an elevated-plus maze (EPM) experiment in male mice. It indeed exerted anxiolytic-like activities after intracerebroventricular (*icv*), intraperitoneal (*ip*) and oral (*po*) administration in an OT-R dependent manner. We analyzed the mediators involved in the anxiolytic-like activities of LM-2 and oxytocin, and found that the

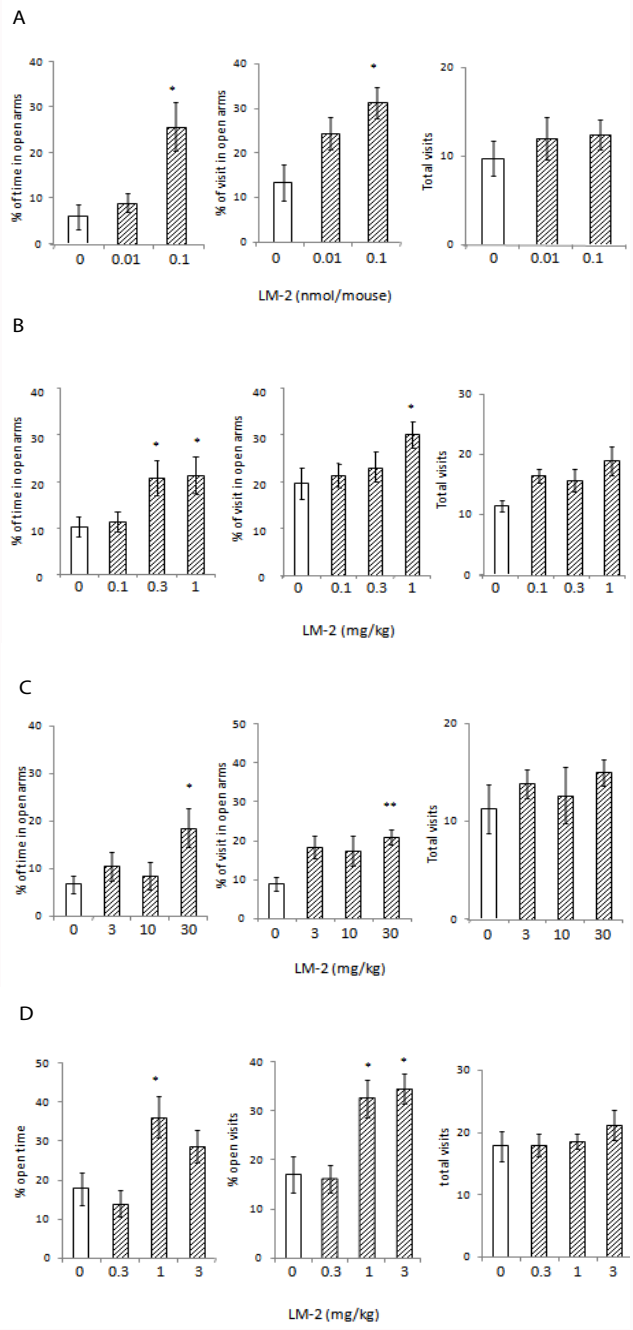

Figure 1: Anxiolytic-like activity of LM-2 in male mice. A: *icv* administration; B: *ip* administration; C: *po* administration; D: *po* administration after emulsification in 30% egg yolk. Peptide was given 20 min before the test for icv administration, and 30 min before the tests for *ip* and *po* administration. Numbers of mice used for individual groups were 7 for A, 9 for B, 8 for C, and 5 for D. Each value is expressed as the mean ± SEM. Significant difference against the parallel control (* $p < 0.05$, ** $p < 0.01$).

adenosine-A_{2A} receptor system is involved downstream of the OT-R, followed by the GABA-$GABA_A$ receptor system.

Materials and Methods

Reagents

LM-2 was synthesized by the Fmoc method and purified by HPLC on an ODS column that was developed by a liner gradient of acetonitrile/water containing 0.1% trifluoroacetic acid. Oxytocin

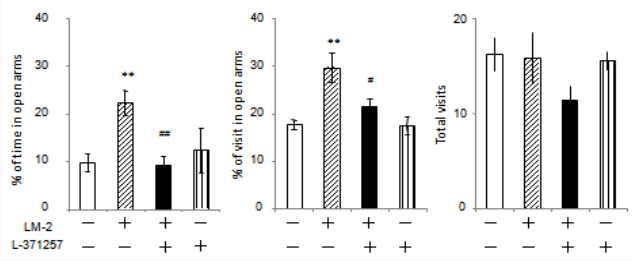

Figure 2: Effect of an OT-R antagonist on anxiolytic-like activity of LM-2 after *icv* administration. L-371,257 (1 nmol/mice), an OT-R antagonist, was co-administered with LM-2 (0.1 nmol/mice) 20 min before the tests. Number of mice used for individual groups was 10. Each value is expressed as the mean ± SEM. Significant difference against the negative control (** $p < 0.01$), and against the positive control ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$).

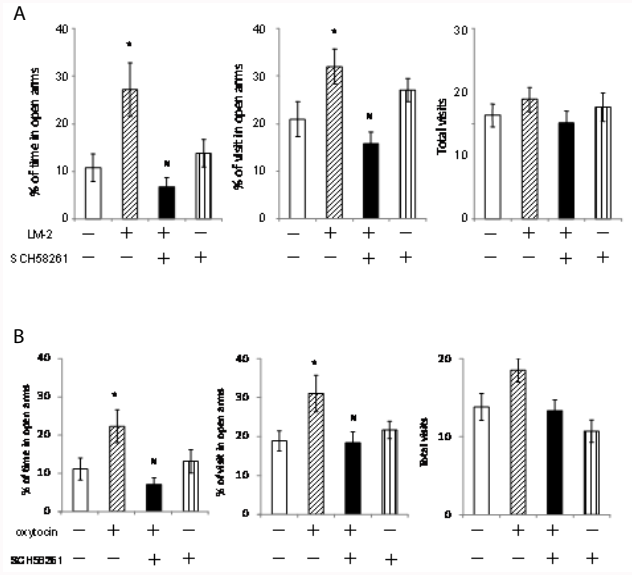

Figure 3: Effect of an adenosine A_{2A} receptor antagonist on anxiolytic-like activities of LM-2 and oxytocin after *icv* administration. A: LM-2 (0.1 nmol/mouse); B. oxytocin (10 nmols/mouse). SCH58261 (1 nmol/mouse), an antagonist for adenosine A_{2A}, was co-administered with peptides (0.1 nmol/mouse) 20 min before the tests. Number of mice used for individual groups was 19 for A, and 7 for B. Each value is expressed as the mean ± SEM. Significant difference against the negative control (* $p < 0.05$), and against the positive control ($^{\#}$ $p < 0.05$).

was obtained from the Peptide Institute (Osaka, Japan). L-371257, SCH58261, and bicuculline were obtained from Tocris (Bristol, U.K.).

Animals

Four-week-old male mice of the *ddY* strain were obtained from SLC Inc. (Shizuoka, Japan). Animals were housed in a temperature controlled room (23°C) on a 12h light-dark cycle. All animals had free access to food pellets and water.

Binding assay for the human OT-R

The binding affinity of LM-2 for the human oxytocin receptor was determined at Panlans Inc. according to the method of Tahara *et al.* using [^{3}H]-oxytocin and Chem-1 cells expressing recombinant human OT-R [14].

Elevated-plus maze experiment

Anxiolytic-like activity was determined using elevated-plus maze

experiments with mice that were 28±2 gr in body weight, as described previously [15]. LM-2 was dissolved in artificial cerebrospinal fluid and injected into the lateral cerebroventricule in a volume of 4μl mouse. LM-2 was dissolved in saline for *ip* and *po* administration.

Statistical analyses

All values are expressed as the mean ± S.E.M. Analysis of variance (ANOVA) followed by Fisher's test was used to assess differences among groups. *P*-values less than 0.05 were considered significant.

Results

The affinity of LM-2 for the human oxytocin receptor

LM-2 showed a weak affinity for the human oxytocin receptor (OT-R) (Ki: 62 μM), despite the fact that its structural homology to oxytocin-CYIQNCPLG-NH_2 is rather small. The Ki value of oxytocin for the OT-R was 0.31 nM under the same conditions.

Anxiolytic-like activity of LM-2 in male mice

As shown in (Figure 1A), LM-2 exerted anxiolytic-like activity after *icv* administration at doses of 0.1 nmol/mouse. After *ip* administration LM-2 showed significant anxiolytic-like activity at doses of 0.3 and 1 mg/kg, as judged by % of time in open arms and % of visit in open arms, respectively (Figure 1B). As shown in (Figure 1C), LM-2 exerted anxiolytic activity at a dose of 30 mg/kg (*po*). We have reported that minimum effective doses after *po* administration of ovokinin and novokinin, anti-hypertensive peptides derived from ovalbumin and its derivative, respectively, became 1/4 and 1/30 those of aqueous solution when given as a emulsion in 30% egg yolk [16,17]. Under the same conditions, LM-2 exerted anxiolytic-like activity at a dose of 1.0 mg/kg, which corresponds to 1/30 that of LM-2 dissolved in saline (Figure 1D).

Effect of an OT-R antagonist on anxiolytic-like activity of LM-2

Mode of action of LM-2 was investigated after *icv* administration. The anxiolytic-like activity of LM-2 was blocked by L-371257, an antagonist of the OT-R (Figure 2). Thus, LM-2 is the first example of an orally effective agonist peptide of the oxytocin receptor. LM-2 is also the first example of an OT-R agonist peptide derived from a natural protein other than preprooxytocin, the endogenous precursor protein.

Effect of an A_{2A} receptor antagonist on the anxiolytic-like activities of LM-2 and oxytocin

We previously reported that the anxiolytic-like activities of endogenous substances such as prostaglandin D_2, complement C5a and formyl peptide receptor 2 (FPR2) agonists were blocked by SCH58261, an A_{2A}-R antagonist, followed by the GABA-$GABA_A$ receptor system [18-20]. We investigated the effect of this antagonist on the anxiolytic-like activities of LM-2 and oxytocin. The anxiolytic-like activity of LM-2 was blocked by SCH58261 (Figure 3A), although LM-2 itself showed no affinity for the A_{2A} receptor. Similarly, the anxiolytic-like activity of oxytocin was also blocked by SCH58261 (Figure 3B). In contrast, the anxiolytic-like activity of the A_{2A}-R agonist CGS2i680 was not blocked by L-371257 (data not shown). These results suggest that the anxiolytic-like activities of LM-2 and oxytocin are mediated by the adenosine-A_{2A} receptor system downstream of the OT-R.

Effect of a $GABA_A$ receptor antagonist on the anxiolytic-like activity of LM-2

The anxiolytic-like activity of oxytocin has been reported to be

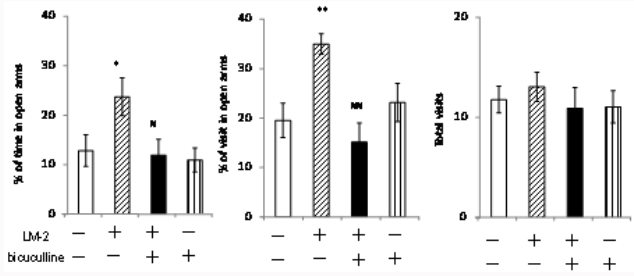

Figure 4: Effect of a $GABA_A$ receptor antagonist on anxiolytic-like activity of LM-2 after *icv* administration. Bicuculline (1nmol/mouse), an antagonist for $GABA_A$ receptor, was co-administered with LM-2 (0.1 nmol/mouse) 20 min before the tests. Number of mice used for individual groups was 15. Each value is expressed as the mean ± SEM. Parentheses represent the number of mice in each group. Significant difference against the negative control (* $p < 0.05$), and against the positive control ($^{\#}p < 0.05$).

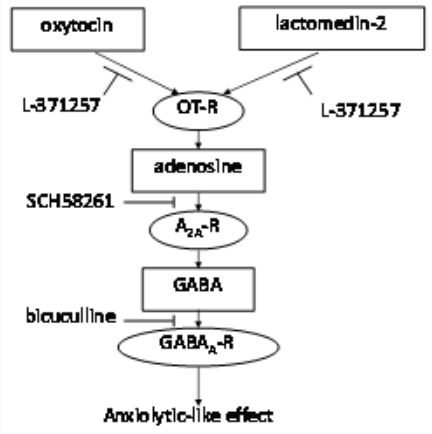

Figure 5: Mechanism for anxiolytic-like activities of oxytocin and LM-2.

mediated by the GABA-$GABA_A$ receptor system since it is blocked by bicuculline, an antagonist of the $GABA_A$ receptor [21]. We investigated the effect of bicuculline on the anxiolytic-like effect of LM-2. As shown in (Figure 4), the effect was blocked by bicuculline, suggesting that it was mediated by the GABA-$GABA_A$ receptor system.

Discussion

The LM-2 derived from human lactoferrin is the first example of an agonist peptide derived from a natural protein other than the usual endogenous precursor protein, preprooxytocin. The LM-2 sequence CFQWQR is found only in human lactoferrin [22]. In chimpanzee lactoferrin, the sequence corresponding to LM-2 is CFRWQR [23]. Because of the presence of the additional Arg residue the 3rd position, which is the cleavage site by trypsin, the corresponding hexapeptide might be cleaved into two tripeptides. In a similar manner, hexapeptides corresponding to LM-2 would not be released from lactoferrin of other species such as mouse, dog, sheep, cow and pig by the action of trypsin [24]. Furthermore, the corresponding sequences are less homologous to oxytocin than LM-2 in these species. Therefore, an agonist peptide of the OT-R seems to most likely can only be released from human lactoferrin.

Although the affinity of LM-2 for the OT-R was found to be very weak, it nevertheless exerted anxiolytic-like activity in male mice

in an OT-R-dependent manner. It should also be noted that LM-2 exerted anxiolytic-like activity at a dose of 0.1 nmol/mouse after *icv* administration, while an oxytocin dose of 1 to 10 nmols/mouse was required by the same route. Many kinds of weak agonist peptides derived from natural proteins have been shown to exert physiological effects despite their affinity for the target receptor being far smaller than that of the endogenous ligands. The smallness of their molecular size and partial resistance to endogenous peptidases may at least partly account for their availability *in vivo*. As for the most probable explanation, LM-2 might be resistant to oxytocinase, cystinyl aminopeptidase, which inactivates oxytocin. Oxytocin is hydrolysed by the enzyme at the Cys^1 residue which is S-S bonded with the Cys^6 residue. In LM-2, however, a peptide bond at the Cys^1 residue might not be hydrolysed by the enzyme because of the presence of a free thiol group. By the way, the Cys^1 residue of LM-2 is essential for its anxiolytic-like activity since [Ala^1]-LM-2 was inactive (unpublished results).

LM-2 exerted anxiolytic-like activity at a dose of 30 mg/kg after *po* administration. When orally given as an emulsion in 30% egg yolk, its minimum effective dose became 1/30 that of aqueous solution. This effect might be caused by facilitation of intestinal absorption of the peptide due to the emulsification. Thus, LM-2 is the first example of an orally effective agonist of the OT-R while oxytocin is known to be effective after nasal administration. LM-2 might serve as a lead compound in the design of orally effective OT-R agonists.

To the best of our knowledge this is the first report that the anxiolytic-like activity of oxytocin, as well as that of LM-2, is mediated by activation of the adenosine-A_{2A} receptor system followed successively by the GABA-$GABA_A$ receptor system downstream of the OT-R.

LM-2 might have activity in helping to reduce mental stress in newborns. Whether LM-2 might thus be effective to some degree in treating anxiety disorders is a point that remains to be tested. It is also an interesting question whether LM-2 possesses the capacity to exert a prosocial effect, including one on mother-infant interactions. Whether LM-2 released in the brain from endogenous lactoferrin by the action of trypsin-like proteases exhibits central effects is yet another issue to be resolved by future investigation.

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